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Fluctuations Between Stabilizing and Destabilizing Electrostatic Contributions of Ion Pairs in Conformers of the c-Myc-Max Leucine Zipper

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ABSTRACT **In solution proteins often exhibit backbone and side-chain flexibility. Yet electrostatic interactions in proteins are sensitive to motions. Hence, here we study the contribution of ion pairs toward protein stability in a range of conformers which sample the conformational space in solution. Specifically, we focus on the electrostatic contributions of ion pairs to the stability of each of the conformers in the NMR ensemble of the c-Myc-Max leucine zipper and to their average energy minimized structure. We compute the electrostatic contributions of inter- and intra-helical ion pairs and of an ion pair network. We find that the electrostatic contributions vary considerably among the 40 NMR conformers. Each ion pair, and the network, fluctuates between being stabilizing and being destabilizing. This fluctation reflects the variability in the location of the ion pairing residues and in the geometric orientation of these residues, both with respect to each other and with respect to other charged groups in the rest of the protein. Ion pair interactions in the c-Myc-Max leucine zipper in solution depend on the protein conformer which is analyzed. Hence, the overall stabilizing (or destabilizing) contribution of an ion pair is conformer population–dependent. This study indicates that free energy calculations performed using the continuum electrostatics methodology are sensitive to protein conformational details. Proteins 2000; 41:485–497.** © 2000 Wiley-Liss, Inc.

Key words: NMR; ensemble; coiled coil; energy landscape; solution

INTRODUCTION

Currently it is still controversial whether ion pairs have stabilizing or destabilizing electrostatic contributions toward protein stability. Experimental as well as calculated estimates of ion pair stabilities indicate that ion pairs can be stabilizing, destabilizing, or insignificant to the overall protein stability.[1–8] We have recently carried out an extensive analysis on a large dataset of structurally nonredundant, high-resolution crystal structures of proteins whose functional form is monomeric.[6] This large-scale analysis has demonstrated that ion pairs can be stabilizing or dstabilizing to the protein, depending on three factors: the buried/exposed location of the ion pairing residues in the protein structure; the distance and geometrical orientation of the side-chain charged groups with respect to each other; and the interaction of the charged groups of the ion pair with the charged groups in the rest of the protein.[6] In particular we have shown that most though not all of the ion pairs adhering to the geometrical definition of a salt bridge, namely a 4.0 Å distance[9] between the charged groups and containing at least a pair of side-chain oxygen and nitrogen atoms within 4.0 Å, are stabilizing to the proteins.

On the other hand, calculations of the contributions of ion pairs in which the geometry is not as optimal show them to be largely destabilizing.[3,6] This has suggested that the stabilizing/destabilizing contributions of ion pairs are sensitive to conformational variability. When analyzing a crystal structure, we are actually analyzing the conformer which is the most favorable for crystallization under these conditions. It does not necessarily represent the conformer with the highest population in solution. As it is being depleted from the solution during crystal growth, the population would shift in its favor, further driving crystal formation.[10–14] Hence, the observation of the sensitivity of the electrostatic contributions of ion pairs to variations in the geometry and in the location leads us directly to questions relating to their stabilizing/destabilizing contributions in populations of conformers in solution.

Abbreviations: C^{α}, Alpha carbon atom of amino acids; Å, Angstrom; Δ, delta; G, Gibb's free energy; Kcal/mol, kilo calories per mole; ASA, accessible surface area; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect.

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Two methods can be used to obtain an ensemble of protein conformations in solution, long molecular dynamics simulations, and nuclear magnetic resonance (NMR) experiments. Currently, molecular dynamic simulations are limited to nanosecond time scales, and multiple long simulations are needed to obtain a reasonable conformational sampling of the ensemble.[15] On the other hand, a typical protein NMR experiment yields an ensemble of conformers staisfying a list of NOE (nuclear Overhauser effect) restraints among hydrogen atoms close in space (1.8 Å–5.0 Å).[16] Dynamic information on proteins is implicit in these restraints and in regions where they are absent. Hence, it is reasonable to expect that ensembles of NMR conformers will provide some information regarding the inherent flexibility of a protein molecule. Recently, Philippopoulos and Lim[17] have compared an ensemble of *E. coli* ribonuclease H1 (RNase H1) conformers derived from NMR experiments both with an ensemble derived from molecular dynamics simulations and with two X-ray structures. They have shown that the 15 conformers of the NMR ensemble sample more conformational space of the RNase H1 than the 1.7 nanosecond molecular dynamics simulations. Furthermore, the NMR average structure corresponds well to the high resolution (1.48 Å) X-ray structure of the RNase H1. MacArthur and Thornton[18] have compared protein structurres derived from NMR data and X-ray crystallography. They found that protein cores observed in NMR structures are well-defined and compare well with those in X-ray structure with resolution of 2.0–2.3 Å. However, there is greater disorder on the surface of NMR-derived protein structures. This may be either due to the inherent flexibility of the protein molecule in solution (as compared to the crystalline state) or due to fewer NOE restraints for surface residues. It is often difficult to separate between the true dynamic behavior of the proteins and artifacts due to incomplete input data and structure calculation protocols. Nevertheless, the space covered by NMR structure ensembles overlaps significantly with that of ensembles generated in long molecular dynamics simulations.[19] Using NMR relaxation methods, Lee et al.[20] have resolved the motion of individual residues between the bound and unbound states of calmodulin and discriminated between backbone and side-chain perturbations.

Here we analyze electrostatic interactions in an ensemble of 40 NMR conformers of the c-Myc-Max leucine zipper solution structure.[21] These conformers can neither be expected to sample the whole conformational space nor do they represent all the conformers of the c-Myc-Max leucine zipper in solution. However, they do reflect, at least to some extent, the protein flexibility around the native state. This is one of a handful of cases for which NMR ensembles containing at least 40 conformers exist in the protein data bank (PDB)[22] (Kumar and Nussinov, unpublished results). It can then be safely concluded that these 40 conformers have significant enough populations in solution to yield NMR signals.

The coiled coil, or the leucine zipper motif, is a supper-secondary structure formed by two or more α-helices winding around each other in a left-handed supercoiled manner. Detailed structural information is available for several leucine zippers.[21,23–25] Both hydrophobic and electrostatic interactions affect the stability and the oligomerization state of the leucine zippers.[26–30] The polar and charged amino acids at positions *e* and *g* in the leucine zipper heptad repeats usually form inter-helical ion pairs. Ion pairs in the GCN4 leucine zipper either do not contribute to stability or may actually be destabilizing.[31,32] Yet at the same time it has also been reported that salt bridges in leucine zippers are stabilizing,[33–36] control the orientation of the α-helices,[37] and impart specificity.[38,39] The c-Myc protein is a member of the proto-oncogene family *myc*. c-Myc and Max preferentially heterodimerize and bind DNA to activate transcription. The heterodimerization domain of the c-Myc-Max complex consists of a parallel coiled coil formed by two α-helices, one each from c-Myc and Max. Recently, the solution structure of a synthetic c-Myc-Max heterodimeric leucine zipper has been determined.[21] The structure has been solved using 2D ^{1}H-NMR. 430 NOE-derived distance restraints have been used for the structure calculations along with additional 15 χ^1 and 50 ψ angle restraints and 50 restraints for backbone hydrogen bonds. Despite the rather small number of restraints, the quality of the 40 conformers in the ensemble is good, especially in the middle region of the structure where most of the restraints are concentrated (pp. 168–170 in reference 21). The c-Myc-Max leucine zipper domain contains four inter- and two intra-helical ion pairs. Two of the four inter-helical ion pairs and the two intra-helical ion pairs are also part of a five-residue ion pair network (IPN-5). All ion pairs and the ion pair network lie in the middle region of the structure. The availability of the NMR ensemble of conformers for the c-Myc-Max leucine zipper allows this study of the roles of intra- and inter-helical ion pairs in the stability of the leucine zipper motif and in the specificity of heterodimer formation.

Using a continuum electrostatics-based methodology,[3] we have computed the electrostatic stabilities of the intra- and inter-helical ion pairs as well as the IPN-5 in the average energy-minimized structure and in the 40 NMR conformers. Our results indicate that the electrostatic strengths of the ion pairs and of the IPN-5 vary considerably in the different NMR conformers. Each of the ion pairs fluctuate between being stabilizing and destabilizing at least once in the 40 conformers. These fluctuations reflect the conformational flexibility of the c-Myc-Max leucine zipper. Furthermore, this study shows the usefulness of the continuum electrostatics methodology in studies of ion pair interactions in solution.

MATERIALS AND METHODS

Intra- and Inter-helical Ion Pairs

All intra- and inter-helical ion pairs proposed from the sequence of the c-Myc-Max leucine zipper have been used

in this study. The following ion pairs contribute to the electrostatic interaction between c-Myc and Max: A Glu 10–B His 13; A Glu 17–B His 13; A Glu 16–B Lys 21; A Glu 17–A Arg 21; A Asp 18–Arg 21; A Arg 23–B Glu 28. Chain A is c-Myc and chain B denotes Max. We have used the residue numbering scheme as in the PDB entries 1a93 and 2a93. NOE peaks were observed for all the above ion pairs.[21] The PDB entry 1a93 contains the average energy-minimized structure and 2a93 contains an ensemble of 40 conformers of c-Myc-Max leucine zipper obtained from the NMR experiment.

Additionally, there is an ion pair network at the c-Myc-Max interface. This network is formed by Glu 10, Glu 17, Arg 21, Asp 18 of c-Myc (chain A), and His 13 of Max (chain B).

Ion Pair Geometry

The geometry of each ion pair is characterized in terms of two quantities:

- The distance (r) between the centroids of the side-chain functional groups in the two charged residues.
- The angular orientation (θ) of the side-chain charged groups in the two ion pairing residues is computed as the angle between two unit vectors, with each unit vector joining a C^{α} atom and the side-chain charged group centroid in a charged residue.[6]

Location of Ion Pairs in the Protein

The locations of residues forming ion pairs were characterized in terms of their solvent accessible surface areas (ASA).[40,41] The probe radius is 1.4 Å. The average of the ASAs of the ion pairing residues indicates the location of the ion pair in the leucine zipper structure. For example, if the ion pairing residues have small ASAs, then the ion pair is largely buried in the protein structure. Conversely, if the ion pairing residues have large ASAs, it indicates that the ion pair is mostly exposed to the solvent (water).

Along with the ion pair geometry, the location of the ion pair is a major determinant of ion pair stability.[6]

Computation of the Electrostatic Energies of Ion Pairs

A detailed protocol for continuum electrostatic calculations has been described previously.[6] Here we have used the same protocol with the exception that there is no need to generate the positions of hydrogen atoms, because we are using NMR data. These calculations are carried out for all the ion pairs listed above, both for the average energy-minimized structure of the c-Myc-Max leucine zipper and for the 40 NMR conformers. The calculations are also performed on the ion pair network, IPN-5, in an analogous manner. All calculations have been carried out at pH 7.0.

Radius of Curvature

Residues Glu 10 through Lys 30 of the c-Myc α-helix and Asn 10 through Gln 30 of the Max α-helix have been used to calculate the radii of curvature. These calculations have been performed using an α-helix geometry characterizing program, HELANAL.[42–44]

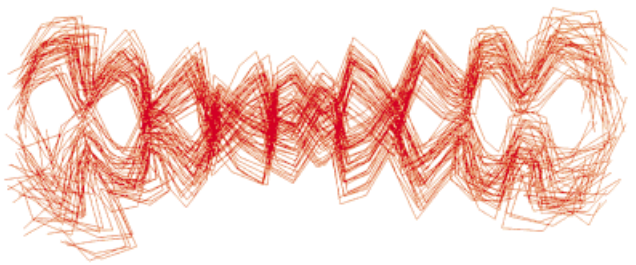

Fig. 1. C^{α} traces in the 40 NMR conformers of the c-Myc-Max leucine zipper. The termini regions show greater disorder than the middle region. All the ion pairs studied here are present in the middle portion of the molecule.

RESULTS

Conformational Variation in the c-Myc-Max Leucine Zipper

NMR data indicate that there is considerable flexibility in the c-Myc-Max leucine zipper domain. Figure 1 presents the C^{α} traces of the 40 NMR conformers. The termini of the α-helices in the leucine zipper have greater disorder due to fewer distance restraints. On the other hand, the middle portion of the leucine zipper helices is well-defined as it has a larger amount of restraints.[21] The radii of the curvature for the c-Myc and Max α-helices vary in different conformers. The mean radius of curvature of the c-Myc α-helix in the 40 NMR conformers (PDB Entry 2a93) is 72 $\pm$ 33 Å and that of the Max α-helix is 65 $\pm$ 24 Å. In the average energy-minimized structure (PDB entry 1a93) of the c-Myc-Max leucine zipper, the radius of curvature of c-Myc α-helix is 121 Å and that of the Max α-helix is 77 Å. Only the middle portions of the c-Myc and Max α-helices have been used in our calculations.

The solution structure of the c-Myc-Max leucine zipper domain reveals the following inter- and intra-helical ion pairs: A Glu 10–B His 13, A Glu 17–B His 13, A Glu 16–B Lys 21, A Glu 17–A Arg 21, A Asp 18–A Arg 21, and A Glu 23–B Arg 28, where chain A is c-Myc and chain B is Max. The residue numbering followed here is that of the PDB[22] files (codes: 1a93 and 2a93). It differs from the one used by Lavigne et al., [21] Based on the sequence, an additional inter-helical ion pair A Arg 21–B Asp 16 was predicted. However, in the solution structure c-Myc Arg 21 was found to be closer to c-Myc Glu 17 and Asp 18, forming the two intra-helical ion pairs listed above. Moreover, residues Glu 10, Glu 17, Asp 18, and Arg 21 from c-Myc, and His 13 from Max form a five-membered ion pair network, IPN-5, which is both intra- and inter-helical. All these ion pairs lie within the better defined middle portions of the helices. The conformations of all ion pairing residues are defined and several NOE signals were observed between residues forming these ion pairs.[21]

Figure 2 plots the ion pair geometries in the 40 NMR conformers. The distances between the side-chain charged

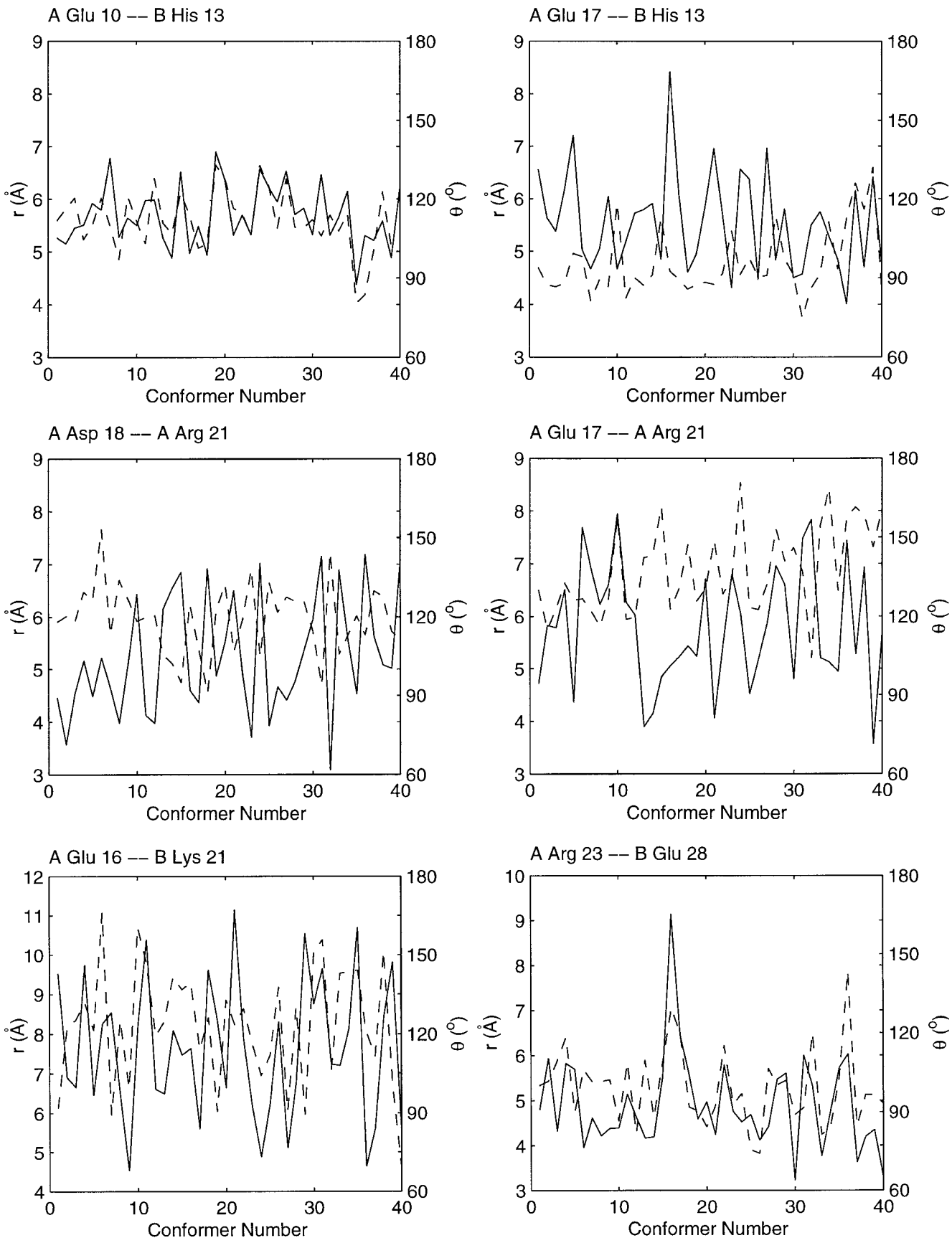
A Glu 10 -- B His 13
A Glu 17 -- B His 13
A Asp 18 -- A Arg 21
A Glu 17 -- A Arg 21
A Glu 16 -- B Lys 21
A Arg 23 -- B Glu 28
r (Å)
θ (°)
Conformer Number

Figure 2.

TABLE I(A). The Ion Pair Geometries in the Average Energy-Minimized Structure of c-Myc-Max Leucine Zipper†

Ion pair	r (Å)	θ (°)
A Glu 10–B His 13	5.75	115
A Glu 17–B His 13	5.67	93
A Glu 17–A Arg 21	5.38	138
A Asp 18–A Arg 21	4.59	124
A Glu 16–B Lys 21	6.77	121
A Arg 23–B Glu 28	4.16	109

TABLE I(B). Location of Ion Pairing Residues in the Average Energy-Minimized Structure of c-Myc-Max Leucine Zipper†

Residue name	Accessible surface area (%)
A Glu 10	53
A Glu 16	52
A Glu 17	52
A Asp 18	40
A Arg 21	57
A Arg 23	58
B His 13	55
B Lys 21	74
B Glu 28	66

†(a) Geometrical orientation and (b) location of the ion pairing residues in the average energy-minimized structure (PDB entry 1a93) of c-Myc-Max leucine zipper. In Table I (a), ion pairs are indicated by the names and numbers of their residues. A denotes c-Myc and B denotes Max. r denotes the distance, in Å, between centroids of side-chain functional groups of the ion pairing residues. θ (in degrees) denotes the angular orientation of the side-chain functional groups in the ion pairs. Refer to Materials and Methods for detailed description of ion pair geometry calculations.

group centroids (r) and the angular orientations of the side-chain charged groups in the ion pairing residues (θ) vary from conformer to conformer. The ion pair geometries in the average energy-minimized structure of c-Myc-Max leucine zipper are given in Table I(a). The accessible surface areas (ASA)[40,41] of the ion pairing residues in the 40 NMR conformers also show extensive variabilities (Fig. 3). The locations of the ion pairing residues, c-Myc Glu 10, Glu 16, Glu 17, Asp 18, Arg 21, and Arg 23 and Max His 13, Lys 21, and Glu 28, are given in Table I(b).

Taken together, these observations indicate that the NMR ensemble of 40 conformers samples a broad conformational space around the native state in solution. The location of ion pairing residues and the ion pair geometries are important determinants of electrostatic strengths of the ion pairs.[6]

In some conformers, side-chain charged groups of a few residues, other than the ion pairs described above, come close enough to be classified as salt bridges, according to the definition of Kumar and Nussinov.[6] These are: A Arg 24–A Glu 25 in conformer 10, A Lys 28–A Glu 32 in conformer 17, A Glu 25–A His 29 in conformer 23, A Lys 22–A Glu 25 in conformer 24, A Lys 12–A Glu 16 in conformer 28, B Asp 18–B Lys 21 in conformer 3, B Asp 19–B Arg 22 in conformers 3 and 17, B Arg 7–B Asp 11 in conformer 38, and A Glu 10–B Lys 9 in conformers 24 and 34. However, Lavigne et al.[21] have not reported NOEs for these residue pairs. Hence, it appears that these salt bridges do not occur with any significant populations in solution. Here, we have not considered these salt bridges in our investigation.

Continum Electrostatics Calculations

The electrostatic stability of each ion pair and of the IPN-5 in all 40 conformers and in the average energy-minimized structure is calculated using the method of computer-mutations of the charged residue side-chains to their hydrophobic isosteres.[3] Hydrophobic isosteres are the charged residue side-chains with their partial atomic charges set to zero. This method has been frequently used in the literature.[4–6,8,45,46] Predictions based on this method have been consistent with experiments.[47]

The total electrostatic free energy contribution $\Delta\Delta G_{tot}$ of an ion pair is given by the following equation:

$$\Delta\Delta G_{tot} = \Delta\Delta G_{dslv} + \Delta\Delta G_{brd} + \Delta\Delta G_{prt}$$

The desolvation energy penalty, $\Delta\Delta G_{dslv}$, is the unfavorable change in energy incurred by ion pairing residues due to the desolvation of the charged side-chains in the folded state of the protein with respect to the unfolded state. This energy term depends on the location of the ion pairing residues in the protein. On average, ion pairs buried in the protein core pay greater desolvation penalties than surface ion pairs.[6] The bridge energy term, $\Delta\Delta G_{brd}$, is due to the electrostatic interaction between the charge groups in ion pairing residue side-chains. This energy term depends on the ion pair geometry as well as on the location.[6] The protein energy term, $\Delta\Delta G_{prt}$, reflects the electrostatic interactions between the charged groups of the ion pair and those in the rest of the protein. This energy term depends on the disposition of the ion pairing residues with respect to the other charges in the protein. There is a weak correlation between the magnitude of this term and the location of the ion pair.[6] We have also computed another thermodynamic quantity called association energy, $\Delta\Delta G_{assoc}$, which represents the stability of the ion pair without taking into account its electrostatic interaction with other charged groups in its environment. It is the free energy change for bringing together oppositely charged side-chains in water relative to their hydrophobic isosteres.[3]

Fig. 2. The geometries of the ion pairs in the c-Myc-Max leucine zipper in the 40 NMR conformers. The Y-axis on the left indicates distance (r, in Å, solid line) between the side-chain charged group centroids in ion pairing residues. The Y-axis on the right indicates angular orientation (θ, in degrees, broken line) of ion pairing residue side chains. The definitions of r and θ are given in the Materials and Methods section. The ion pair corresponding to each plot is shown above the plot.

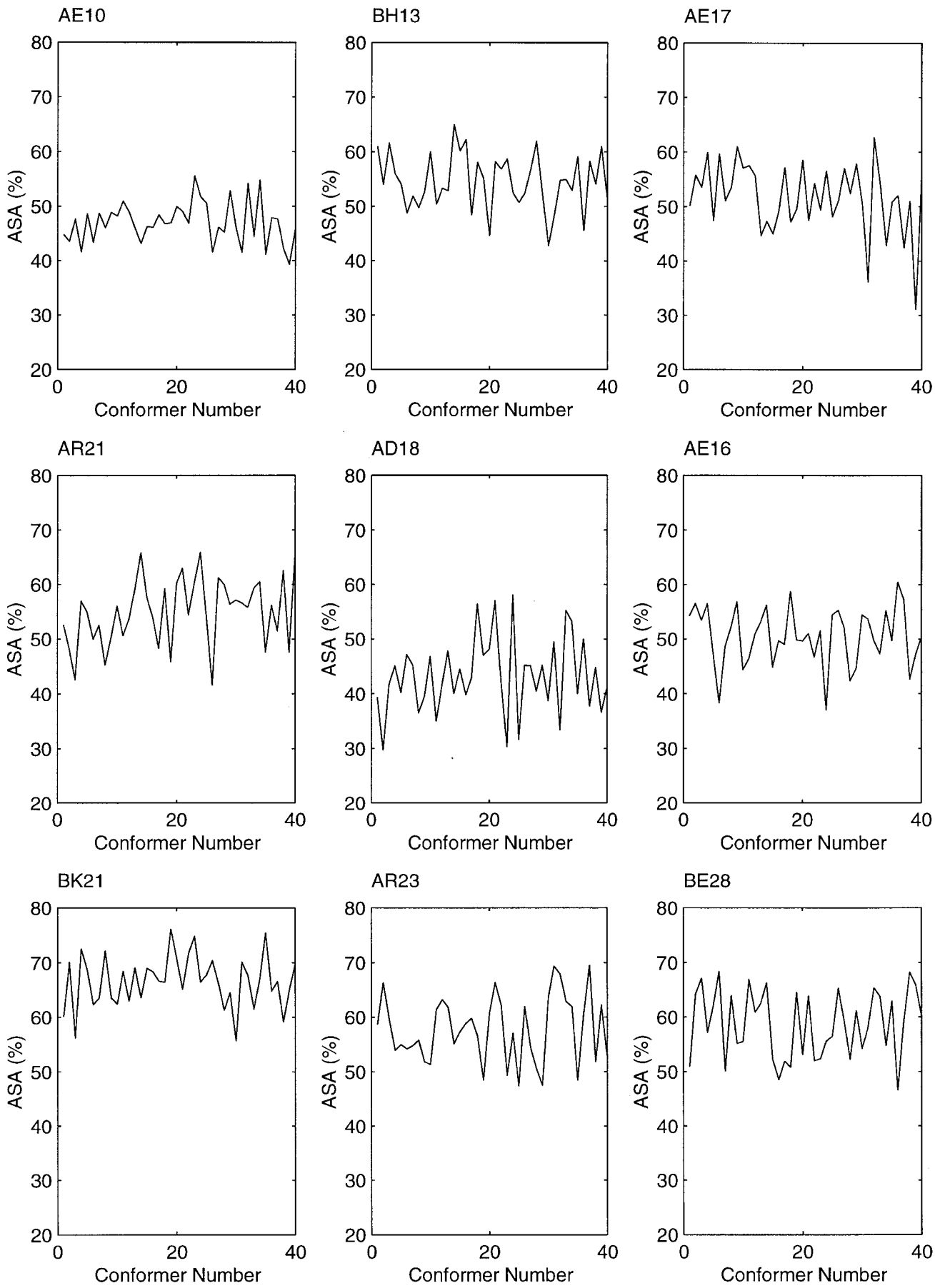
AE10
BH13
AE17
AR21
AD18
AE16
BK21
AR23
BE28
ASA (%)
Conformer Number
20
30
40
50
60
70
80
0
20
40

Figure 3.

TABLE II(A). Electrostatic Contribution of Ion Pairs and IPN-5 in the Average Energy-Minimized Structure of the c-Myc-Max Leucine Zipper†

Ion pair	$\Delta\Delta G_{dslv}$ (Kcal/mol)	$\Delta\Delta G_{brd}$ (Kcal/mol)	$\Delta\Delta G_{prt}$ (Kcal/mol)	$\Delta\Delta G_{tot}$ (Kcal/mol)	$\Delta\Delta G_{assoc}$ (Kcal/mol)
AE10–BH13	+6.81	−1.72	−2.24	+2.85	−0.64
AE17–BH13	+7.14	−1.93	−0.34	+4.87	−0.81
AE17–AR21	+3.74	−1.67	+0.52	+2.59	−1.05
AD18–AR21	+1.23	−0.95	−1.87	−1.59	−0.68
IPN-5[a]	+8.39	−4.87	+0.89	+4.41	−2.51
AE16–BK21	+1.03	−0.77	+0.02	+0.28	−0.52
AR23–BE28	+2.00	−3.15	+0.29	−0.85	−2.41

TABLE II(B). The Minimum and Maximum Values of Various Free Energy Terms for Ion Pairs and IPN-5 in the 40 NMR Conformers of c-Myc-Max Leucine Zipper†

	$\Delta\Delta G_{dslv}$ (Kcal/mol)		$\Delta\Delta G_{brd}$ (Kcal/mol)		$\Delta\Delta G_{prt}$ (Kcal/mol)		$\Delta\Delta G_{tot}$ (Kcal/mol)		$\Delta\Delta G_{assoc}$ (Kcal/mol)	
Ion pair	Min	Max	Min	Max	Min	Max	Min	Max	Min	Max
AE10–BH13	+4.32	+14.43	−6.70	−0.87	−7.67	−0.55	−1.16	+9.70	−2.13	−0.33
AE17–BH13	+6.18	+13.93	−7.71	−0.75	−4.40	+2.73	−2.27	+8.49	−4.51	−0.35
AE17–AR21	+2.65	+12.38	−5.83	−0.60	−6.35	+3.47	−1.38	+8.28	−3.98	−0.09
AD18–AR21	+0.42	+4.00	−3.25	−0.60	−7.60	−0.39	−5.69	+1.53	−2.92	−0.42
IPN-5[a]	+7.18	+18.16	−13.35	−3.14	−2.56	+5.46	−1.57	+14.33	−6.39	−1.14
AE16–BK21	+0.48	+4.53	−1.81	−0.37	−2.24	+1.20	−0.81	+3.82	−1.13	−0.22
AR23–BE28	+1.11	+6.36	−8.10	−0.53	−2.05	+1.03	−6.03	+3.94	−6.90	−0.32

†Ion pairs are indicated by the names and numbers of their residues. Residues names are in single letter codes. A denotes c-Myc and B denotes Max. For each ion pair, $\Delta\Delta G_{dslv}$ denotes the desolvation energy penalty paid by the ion pairing residues, $\Delta\Delta G_{brd}$ indicates the electrostatic interaction energy between the ion pairing residues, $\Delta\Delta G_{prt}$ represents the electrostatic interaction energy between the ion pair and the other charges in the rest of the protein. Total electrostatic free energy of the ion pair is given by $\Delta\Delta G_{tot}$. $\Delta\Delta G_{assoc}$ denotes the association energy of the ion pair. The various free energy terms have been described in the text. In Table II(B), Min stands for minimum value and Max stands for maximum value.

[a]Ion pair network formed by five charged residues, namely, c-Myc Glu 10, Glu 17, Asp 18, Arg 21, and Max His 13.

TABLE II(C). Number of Conformers for Which Ion Pairs and IPN-5 Are Stabilizing or Destabilizing

Ion pair	Stabilizing	Destabilizing
AE10–BH13	1	39
AE17–BH13	2	38
AE17–AR21	6	34
AD18–AR21	37	3
IPN-5†	1	39
AE16–BK21	8	32
AR23–BE28	10	30

In the previous section we have shown that geometries and locations of all the ion pairs vary in different conformers of the c-Myc-Max leucine zipper. Hence, it is reasonable to expect that the various energy terms would fluctuate in the conformer ensemble of c-Myc-Max leucine zipper (2a93). Tables II(a) presents the values of these terms in the average energy-minimized structure (1a93) and Table II(b) presents the minimum and maximum values of these energy terms in the 40 NMR conformers (2a93) of c-Myc-Max leucine zipper. Figures 4(a) and 4(b) show that the total electrostatic free energies of the ion pairs and of the IPN-5 in the c-Myc-Max le4ucine zipper vary considerably in the 40 NMR conformers, with each pair interchanging between being stabilizing and destabilizing at least once. The number of conformers for which the ion pair is stabilizing, or destabilizing, is listed in Table II(c). Below we describe the behavior of IPN-5 and of each ion pair in the c-Myc-Max leucine zipper conformer ensemble.

Fig. 3. Variations in the location of the charged residues in the ion pairs and IPN-5 in the 40 NMR conformers of the c-Myc-Max leucine zipper. The residue corresponding to each plot is shown at its upper left corner. Residue names are given in the standard single letter code. Chains A and B stand for c-Myc and Max, respectively. In each plot, the Y-axis indicates accessible surface area (ASA) and the X-axis indicates the conformer number. The location of each charged residue fluctuates among the conformers.

Ion Pair Network, IPN-5

The unusual presence of His 13 (instead of an apolar residue) at position *d* in the Max α-helix is thought to be responsible for the specificity in heterodimerization between the c-Myc and Max α-helices.[21] The conformation of the B (Max) His 13 is well-defined with more than 40 NOE restraints. This histidine interacts with two glutamates in the c-Myc α-helix, Glu 10, and Glu 17 at position *a* in successive heptad repeats. In addition, B His 13 also interacts with B (Max) Asn 10 and packs between A (c-Myc) Leu 13 and Ile 14.[21] A Glu 17 further forms an intra-helical ion pair with A Arg 21, which in turn inter-

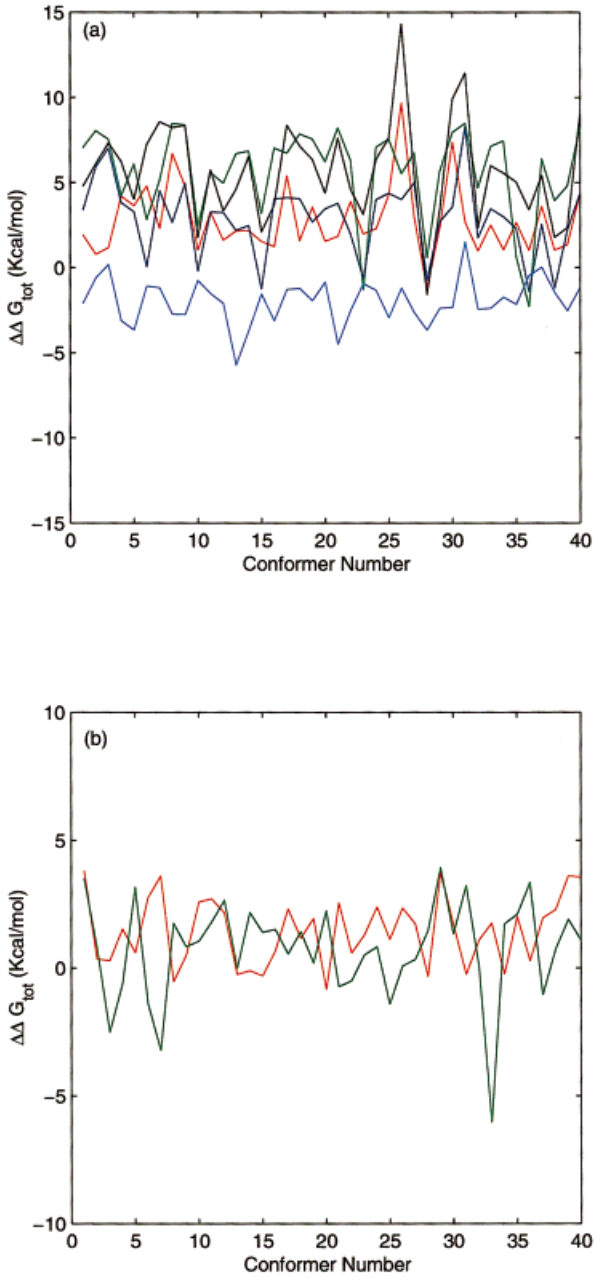

Fig. 4. The variation in the total electrostatic free energies for (**a**) the five residue ion pair network, IPN-5, and its constituent ion pairs, and (**b**) the two isolated ion pairs in the NMR conformer ensemble of the c-Myc-Max leucine zipper. In each plot, the Y-axis represents the total free energy $\Delta\Delta G_{tot}$ and the X-axis indicates the conformer number. Identities of the ion pairs and of the IPN-5 are color coded. In a, black color indicates the ion pair network IPN-5. Blue and cyan colors are for the intra-helical ion pairs A Asp 18–A Arg 21 and A Glu 17–A Arg 21, respectively. Red and green colors denote the ion pairs likely to be responsible for the heterodimer specificity, A Glu 10–B His 13, and A Glu 17–B His 13, respectively. In b, red and green colors denote isolated inter-helical ion pairs A Glu 16–B Lys 21 and A Arg 23–B Glu 28, respectively. Note that each ion pair and the ion pair network fluctuates between being stabilizing and being destabilizing at least once.

acts with A Asp 18. Hence, c-Myc Glu 10, Glu 17, Asp 18, Arg 21, and Max His 13 form an ion pair network in the c-Myc-Max leucine zipper heterodimer. The four component ion pairs of this network, two inter-helical ion pairs A Glu 10–B His 13 and A Glu 17–B His 13, and two intra-helical ones A Glu 17–A Arg 21 and A Asp 18–A Arg 21, are described below in separate sections.

IPN-5 is destabilizing in 39 our of the 40 NMR conformers. In most conformers, the total electrostatic contribution of IPN-5 $\Delta\Delta G_{tot-network}$ is +3 to +9 Kcal/mol. In the average energy-minimized structure it is destabilizing, with $\Delta\Delta G_{tot-network}$ +4.41 Kcal/mol (Table II(a)). IPN-5 plays a large desolvation penalty ($\Delta\Delta G_{dslv-network}$ = +7 to +13 Kcal/mol) in most of the conformers. This large desolvation penalty is often not compensated by the bridging interactions in the IPN-5 side-chain charged groups. $\Delta\Delta G_{brd-network}$ varies between −3 to −9 Kcal/mol in most of the conformers. $\Delta\Delta G_{prt-network}$ is destabilizing in 28 conformers and stabilizing in the remaining 12. It fluctuates between −2 to +5 Kcal/mol.

In conformer 28, where IPN-5 is stabilizing, $\Delta\Delta G_{tot-network}$ is −1.6 Kcal/mol. In this conformer, the IPN-5 pays a relatively smaller desolvation penalty $\Delta\Delta G_{dslv-network}$ of +8.2 Kcal/mol, which is overcome by the stronger electrostatic interactions among the IPN-5 residue side-chain charged groups. $\Delta\Delta G_{brd-network}$ is −10.1 Kcal/mol in this conformer. $\Delta\Delta G_{prt-network}$ is +0.3 Kcal/mol in this conformer. In conformer 28, three out of the four component ion pairs, namely, A Glu 10–G His 13, A Glu 17–A Arg 21, and A Asp 18–A Arg 21 are also stabilizing. The fourth ion pair, A Glu 17–B His 13, is only weakly destabilizing by +0.6 Kcal/mol.

Inter-helical Ion Pairs A Glu 10–B His 13 and A Glu 17–B His 13

In the average energy-minimized structure, both of these ion pairs are destabilizing (positive $\Delta\Delta G_{tot}$). The burial of the residues forming these ion pairs costs large desolvation energy penalities ($\Delta\Delta G_{dslv}$) that are not overcome by the favorable electrostatic interactions between the residues in the ion pairs ($\Delta\Delta G_{brd}$) and between the ion pairs and the charges in the rest of the leucine zipper ($\Delta\Delta G_{brd}$) and between the ion pairs and the charges in the rest of the leucine zipper ($\Delta\Delta G_{prt}$) (Table II(a)). In the majority of the conformers, the ion pair A Glu 10–B His 13 incurs a desolvation penalty of +5 to +10 Kcal/mol. $\Delta\Delta G_{brd}$ varies between between −1 to −4 Kcal/mol, and $\Delta\Delta G_{prt}$ fluctuates between −1 to −5 Kcal/mol for this ion pair in most conformers. The protein energy term is stabilizing in all 40 conformers for this ion pair. In the average energy-minimized structure, its $\Delta\Delta G_{tot}$ = +2.85 Kcal/mol (Table II(a)).

Among the 40 conformers, the ion pair A Glu 10–B His 13 is stabilizing (negative $\Delta\Delta G_{tot}$) only once, in conformer 28. This is owing to an improved electrostatic interaction of the ion pair with the rest of the leucine zipper in this conformer. Additionally, in this conformer the ion pair pays a relatively small desolvation penalty. B His 13 is

more solvent exposed, with an accessible surface area of 62%.

The ion pair A Glu 17–B His 13 incurs a desolvation penalty, $\Delta\Delta G_{dslv}$, of +5 to +11 Kcal/mol in most of the NMR conformers. This desolvation penalty is countered by the bridge energy term $\Delta\Delta G_{brd}$ of −1 to −5 Kcal/mol. The protein energy term for this ion pair is destabilizing for 25 conformers and stabilizing for the remaining 15. In the majority of the cases, the protein energy term $\Delta\Delta G_{prt}$ fluctuates between −1 Kcal to +2 Kcal/mol. In the average energy-minimized structures, its $\Delta\Delta G_{tot}$ = + 4.87 Kcal/mol (Table II(a)).

This ion pair is stabilizing in two of the 40 conformers (conformer numbers 23 and 36). The interaction between the charged side-chains (bridge energy term) improves considerably in these conformers. Side-chain centroids between the two residues are closer (4.3 Å in conformer 23 and 4.0 Å in conformer 36 as compared to 5.7 Å in the average energy-minimized structure). The protein energy term also improves considerably in these two conformers. In conformer 36, $\Delta\Delta G_{tot}$ for this ion pair is stabilizing by −2.27 Kcal/mol. Both the bridge and protein energy terms are quite strong ($\Delta\Delta G_{brd}$ = − 7.71 Kcal/mol and $\Delta\Delta G_{prt}$ = − 4.4 Kcal/mol), indicating good electrostatic interactions among charges within the ion pair and between it and the rest of the protein.

Intra-helical Ion Pairs A Glu 17–A Arg 21 and A Asp 18–A Arg 21

The ion pair A Glu 17–A Arg 21 incures a desolvation penalty $\Delta\Delta G_{dslv}$ of +3 and +7 Kcal/mol in most of the NMR conformers. The electrostatic interaction between the side-chain charged groups of Glu 17 and Arg 21 is weak, with $\Delta\Delta G_{brd}$ varying between −0.5 and −2.5 Kcal/mol. The protein energy term for this ion pair shows a bimodal behavior. $\Delta\Delta G_{prt}$ for this ion pair is stabilizing in 22 conformers and destabilizing in the other 18. When destabilizing, the protein energy term has a magnitude of 0 to +2 Kcal/mol, and when stabilizing, it varies between −1 to −4 Kcal/mol. In the average energy-minimized structure, the protein term is destabilizing by +0.5 Kcal/mol. Overall, this ion pair is destabilizing ($\Delta\Delta G_{tot}$ = + 1 to +5 Kcal/mol) in 34 conformers and in the average energy-minimized structure. In the average energy-minimized structure, $\Delta\Delta G_{tot}$ for this ion pair is +2.59 Kcal/mol. The A Glu 17–A Arg 21 ion pair is stabilizing in six conformers mainly due to its improved electrostatic interactions with its protein neighborhood (protein energy term). Only in three out of these six conformers (numbers 15, 36, and 38) does its stabilizing contribution becomes marginally stronger than −1 Kcal/mol.

In contrast, the intra-helical ion pair formed between c-Myc Asp 18 and Arg 21 is stabilizing in 37 out of the 40 conformers. In the average energy-minimized structure, this ion pair contributes −1.6 Kcal/mol. $\Delta\Delta G_{brd}$ for this ion pair varies between −0.5 to −2.0 Kcal/mol. $\Delta\Delta G_{dslv}$ incurred by this ion pair is +0.5 to +2.0 Kcal/mol. The protein energy term for this ion pair is stabilizing in all conformers, and varies between −0.5 to −2.5 Kcal/mol. $\Delta\Delta G_{tot}$ fluctuates between −1 to −3 Kcal/mol in most of the conformers.

This ion pair is destabilizing in three conformers. In two of these conformers (conformers 3 and 37), the $\Delta\Delta G_{tot}$'s for this ion pair are only +0.2 Kcal/mol and +0.03 Kcal/mol, respectively. In the remaining conformer (conformer 31), $\Delta\Delta G_{tot}$ is +1.53 Kcal/mol. This ion pair pays a relatively higher desolvation penalty of +3.18 Kcal/mol. This desolvation penalty is not overcome by the weak bridge and protein energy terms. In this particular conformer, the distance between the side-chain charged group centroids of A Asp 18 and A Arg 21 increases to 7.1 Å (as compared to 4.6 Å in the average energy-minimized structure).

Isolated Inter-helical Ion Pairs A Glu 16–B Lys 21 and A Arg 23–B Glu 28

The c-Myc-Max leucine zipper has two isolated inter-helical ion pairs, A Glu 16–B Lys 21 and A Arg 23–B Glu 28, which are not part of any ion pair network. These isolated ion pairs also fluctuate between being stabilizing and destabilizing to the c-Myc-Max leucine zipper. In these ion pairs the fluctuations are more frequent than those that are part of the network, and their total electrostatic contributions are smaller. Interestingly, there is little interaction between these ion pairs and the charged groups in the rest of the leucine zipper.

In the average energy minimized structure, ion pair A Glu 16–B Lys 21 is marginally destabilizing, with $\Delta\Delta G_{tot}$ being +0.28 Kcal/mol. $\Delta\Delta G_{dslv}$ is +1.03 Kcal/mol, $\Delta\Delta G_{brd}$ is −0.77 Kcal/mol, and $\Delta\Delta G_{prt}$ is +0.02 Kcal/mol. Thus, the electrostatic interactions both between the ion pairing side-chains and between the ion pair and the charges in the protein environment are weak. In the average energy-minimized structure, the side-chain charged group centroids are 6.8 Å apart, and are oriented at an angle at 121° (Table I(a)). In most conformers, this ion pair pays small desolvation penalties, $\Delta\Delta G_{dslv}$, of +0.5 to +3.5 Kcal/mol. $\Delta\Delta G_{brd}$ varies between −0.25 to −1.5 Kcal/mol and $\Delta\Delta G_{prt}$ varies between −1 to +1 Kcal/mol. The protein energy term is stabilizing in 20 out of the 40 NMR conformers. The ion pair exhibits mostly a bimodal behavior, with $\Delta\Delta G_{tot}$ varying between −0.5 to +2.5 Kcal/mol. It is destabilizing in 32 conformers. In seven (out of the remaining eight) conformers, the ion pair becomes stabilizing owing to a drop in the desolvation penalties. Max Lys 21 is more solvent-accessible in these conformers, with its ASA ~70%. The protein energy terms are weakly stabilizing and the bridge energy terms remain weak. In the remaining conformer (conformer 28), this ion pair pays a higher desolvation penalty ($\Delta\Delta G_{dslv}$ = + 2.75 Kcal/mol). In this conformer both c-Myc Glu 16 and Max Lys 21 have relatively lower ASAs, *i.e.,* they are less accessible to the solvent. The bridge energy term $\Delta\Delta G_{brd}$ is only −0.82 Kcal/mol. However, the ion pair has a strong protein energy term $\Delta\Delta G_{prt}$ = − 2.24 Kcal/mol. $\Delta\Delta G_{tot}$ for this ion pair in all eight conformers is weaker than −1 Kcal/mol. In the ion pair A Glu 16–B Lys 21, the observed NOE

data are ambiguous due to spectral overlap of protons in A Glu 16.

Several NOE signals exist between the two residues forming the ion pair A Arg 23–B Glu 28.[21] This ion pair is located relatively near the C-terminal region of the leucine zipper. In the average energy-minimized structure, this ion pair is (weakly) stabilizing, with $\Delta\Delta G_{tot}$ − 0.85 Kcal/mol. It pays a desolvation penalty $\Delta\Delta G_{dslv}$ of +2.00 Kcal/mol, which is easily overcome by the bridge energy term $\Delta\Delta G_{brd}$ of −3.15 Kcal/mol, despite the slightly destabilizing protein energy $\Delta\Delta G_{prt}$ (+ 0.29 Kcal/mol). In the average energy-minimized structure, this ion pair has a reasonable geometry. The side-chain centroids of c-Myc Arg 23 and Max Glu 28 are 4.16 Å apart and the side-chain charged group orientation angle is 109.2° (Table I(a)). However, this ion pair is destabilizing in 30 out of the 40 NMR conformers. In the 40 conformers, this ion pair has an average $\Delta\Delta G_{tot}$ of +0.8 ± 1.9 Kcal/mol. The protein energy term is stabilizing in 17 conformers and destabilizing in 23. In most conformers, this ion pair pays desolvation penalties $\Delta\Delta G_{dslv}$ of +1.5 to +4.5 Kcal/mol. The bridge energy term $\Delta\Delta G_{brd}$ fluctuates between −1 to −5 Kcal/mol, and the protein energy term $\Delta\Delta G_{prt}$ fluctuates between −1.5 to +1.5 Kcal/mol. $\Delta\Delta G_{tot}$ varies between −3.0 and +5 Kcal/mol in most conformers.

In conformers 3, 6, 7, 25, 33, and 37 this ion pair is relatively stabilizing with $\Delta\Delta G_{tot}$ stronger than −1 Kcal/mol. Interestingly, this ion pair is quite strong ($\Delta\Delta G_{tot}$ = − 6.03 Kcal/mol) in conformer 33. In this conformer, there is a strong electrostatic interaction between the ion pairing side-chain charged groups ($\Delta\Delta G_{brd}$ = − 8.10 Kcal/mol). The desolvation penalty $\Delta\Delta G_{dslv}$ is +2.12 Kcal/mol, and the protein energy term $\Delta\Delta G_{prt}$ is −0.05 Kcal/mol in this conformer. The distance between the ion pairing residue side-chain centroids is 3.77 Å and the charged groups orientation angle is 81.4°. Here, the c-Myc Arg 23 guanidium nitrogen atoms N^{ε} and $N^{\eta 2}$ are within hydrogen bonding distances from the Max Glu 28 side-chain carbonyl oxygen atoms $O^{\varepsilon 1}$ and $O^{\varepsilon 2}$. As a result, four side chain-side chain hydrogen bonds are formed within this ion pair in conformer number 33.

DISCUSSION

The rationale for using an NMR ensemble rather than a crystal structure or an averaged energy-minimized structure, is that it reflects to a certain extent that conformational flexibility of the molecule. In solution, a protein exists as an ensemble of conformational isomers, with different population times.[10,11,13] Hence, the stabilizing (or destabilizing) effect of an interaction depends on the population time of the conformer in which it occurs. Depending on the physical or the binding condition of the protein, the energy landscape and the populations may shift.[12,14] Concomitantly, there may be a change in the stabilizing (or destabilizing) contribution of the ion pair.

Traditionally, it has been debated whether a conformational ensemble obtained by NMR experiments truly reflects the dynamics of protein behavior in solution. The NMR conformational ensemble contains different structures that are compatible with the existing and the missing NOE restraints.[16] However, frequently only partial datasets are available. This along with procedures followed in the structural assignment may lead to difficulties in distinguishing and in quantifying differences between the true dynamic behavior of the molecule and inaccuracies in the structure determination.[17] In spite of these difficulties, it is clear that at least part of the conformational variability of NMR structures arises from protein motion. The local disorder in NMR structural ensembles has been compared with crystallographic temperature factors. The percentage of charged residue side-chains exhibiting greater than average disorder is similar between ensembles of NMR structures and crystallographic B-factors.[48] Heteronuclear NMR relaxation experiments are useful in studying and overcoming ambiguities in NMR data due to protein motion.[49] There are a number of reports in the literature that compare NMR structural ensembles with those derived from molecular dynamic simulations. These reports provide convincing evidence that NMR conformational ensembles reflect protein dynamics in solution (for example, 17, 19, and references therein). Hence, NMR is an important tool for studying protein dynamics and for the characterization of states not accessible to X-ray crystallography.[50] In recent years, NMR has been widely used to study protein mobility.[20,51]

The positions and orientations of charged residues vary with respect to each other in solution. Our recent studies on salt bridges have shown that their electrostatic strengths depend on the location of the charged residues, the orientation of the side-chain charged groups with respect to one another, and their interactions with other charges in the proteins.[6] Hence, while the observed variations in ion pair stabilities are not surprising, this study provides some indication of the extent of the variations that can occur in the stabilities of ion pairs in solution. In this regard, it is interesting to note that all ion pairs and the ion network IPN-5 interconvert between being stabilizing and destabilizing in the NMR conformer ensemble of c-Myc-Max leucine zipper, providing a glimpse into the electrodynamic interactions in this heterodimer in solution. The validity of the results presented here depends upon the quality of the NMR data. We cannot rule out errors in our calculations resulting from inherent ambiguities in the available NMR data and from potential artifacts in structure calculation protocols. We have been careful to study only those ion pair interactions which are based on the NOE restraints and which fall within the better defined middle portion of the structure. In the average energy-minimized structure and in most conformers, the side-chain charged group centroids for the residues in most ion pairs lie outside the 4.0 Å distance limit.[9] In the average energy-minimized structure, side-chain charged group centroids fall within the 4.0 Å distance for only two ion pairs, namely, A Asp 18–A Arg 21 and A Arg 23–Glu 28 (Table I(a)). Hence, the ion pair geometries (defined in the Materials and Methods section) observed here are porrer

than those of the salt bridges analyzed in the high-resolution crystal structure study.[6] Using ^{1}H NMR, it is difficult to pin point ion pair geometries since Asp and Glu side-chain carboxyl groups (COO^-) lack hydrogen atoms.

In spite of these limitations, the errors involved are likely to be small and as such are not expected to affect the overall conclusions of this study. To further probe the observed trends, we have carried out a statistical analysis of ion pair interactions in NMR conformer ensembles of 11 different proteins. The results of this large analysis have confirmed our observations on the ion pairs of c-Myc-Max leucine zipper (Kumar and Nussinov, unpublished results). Our study further indicates that the continuum electrostatics methodology used to compute free energies of the ion pairs is sensitive to the detailed molecular conformation.

Previously, Lavigne et al.[52] have shown that the stability of the c-Myc-Max leucine zipper heterodimer is pH dependent, with the heterodimer being maximally stable around pH 4.5. At 25°C the pK_a of Max His 13 increases by 0.42 pK_a units in the folded state as compared to the unfolded state. This observation indicates that Max His 13 side-chain stabilizes the heterodimer by approximately 0.57 Kcal/mol. This stabilization is thoguht to originate in ion pairs formed by Max His 13 with c-Myc Glu 10 and Glu 17.[21,52] On the other hand, our calculations show these ion pairs and the IPN-5 to be destabilizing in most, though not in all, of the NMR conformers and in the average energy-minimized structure. This raises the question as to how to understand this difference between the experimental and the computational results. There are several potential reasons for this apparent disagreement.

To begin with, the experimental estimate of the electrostatic contribution of Max His 13 toward the stability of the folded state of c-Myc-Max heterodimer is small. This may suggest that the role of Max His 13 is largely to contribute toward specificity in heterodimer formation rather than to contribute toward its stability. Such an interpretation appears to be consistent with the experimental results on the GCN4 leucine zipper. Lumb and Kim[31] have shown that interhelical ion pairs do not contribute to the stability of the leucine zipper. They have further shown that a buried polar interaction imparts structural specificity to a designed, GCN4-based heterodimeric leucine zipper, at the expense of stability.[39] The small electrostatic contribution of His 13 toward the folded-bound state of c-Myc-Max leucine zipper indicates that the electrostatic contribution of His 13 may actually fluctuate between being stabilizing and destabilizing in the different conformers in solution. Our calculations show that this is indeed the case. However, there is no information available on the relative conformer populations in solution.

At pH 7.0, nearly 50% of Max His 13 is protonated (pK_a for Max His 13 is 7.2 in the folded state),[52] and the strength of the interaction of Max His 13 with c-Myc Glu 10 and Glu 17 is expected to be weak,[21] as shown by the bridge terms in our calculations performed at this pH. However, at lower pH (~5.0) all of Max His 13 are protonated. This would facilitate stronger interactions between Max His 13 and c-Myc Glu 10 and Glu 17. This may be partly responsible for the pH dependence of c-Myc-Max leucine zipper peptide stability.[21] The energy landscapes of proteins and peptides are dynamic, changing with environmental conditions such as temperature, pH, and the presence (or absence) of ligands.[14] At pH 5.0, the energy landscape of c-Myc-Max heterodimer may shift with respect to that at pH 7.0. The populations of conformers containing the stabilizing ion pairs and the ion pair network involving Max His 13 may increase, consistent with the experimental observations.

The advantages and limitations of the method used in the calculations have been discussed in detail by Hendsch and Tidor.[3] Similarly, the advantages and limitations of experimental methods and the differing interpretation of experimental results regarding the contribution of interhelical ion pairs to the stability of coiled coils and to the specificity of dimerization have also been discussed in an interesting correspondence between Lavigne et al.[53] and Lumb and Kim.[32] As the authors have noted, the pK_a value of an ionizable group in a protein depends on several factors such as temperature, ionic strength, and its microenvironment. An increase in the pK_a value of an ionizable group in the folded state of a protein does not always indicate a stabilizing electrostatic contribution by the ionizable group. We further note that the method we employ works by carrying out a computer mutation of charged residue side-chains to their hydrophobic isosteres.[3,6] Unfortunately, experimentally there is no way to switch off partial atomic charges on side-chains. Hence, the reference states used in the calculations and in the experiments may differ.

CONCLUSIONS

The overall stability of an ion pair is a statistical outcome of the relative populations of the conformers of the protein. Here we have shown this principle for one type of motif, the coiled coil. Our current large-scale electrostatic energy calculations of salt bridges in NMR conformer ensembles of a broad range of different proteins support these observations (Kumar and Nussinov, unpublished results).

The electrostatic contributions of each ion pair and of the IPN-5 are *conformer-dependent*. Furthermore, each ion pair and the IPN-5 fluctuates between being stabilizing and being destabilizing at least once. This fluctuation is due to the variability in the factors that determine the electrostatic contribution of an ion pair. These variabilities are, in turn, the outcome of the flexibility of the c-Myc-Max leucine zipper domain. The solution structure of the c-Myc-Max leucine zipper has yielded a fairly broad ensemble of conformers around the native state. The α-helices still show extensive variations in the overall curvatures, along with side-chain flexibilities.

Our calculations support the premise that ion pairs, especially the inter-helical ones, may not contribute significantly toward the thermodynamic stability of the c-Myc-

Max leucine zipper. Instead, these interactions may be responsible for the specificity of heterodimer formation. All proteins that interact with Max via a leucine zipper domain contain a conserved acidic residue capable of ion pair formation with His 13 in Max,[21] indicating its critical role in heterodimer specificity. These observations are in agreement with some experiments[31,32,39] though not with all.[52,53] Furthermore, in a recent study on the vibrational properties of the c-Myc-Max leucine zipper and its monomeric constituents, it has been observed that the heterodimer has significantly less vibrational entropy as compared to the c-Myc and the Max monomers alone.[54]

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